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(54) Title: METHODS FOR MULTI-STAGE SOLID PHASE AMPLIFICATION OF NUCLEIC ACIDS

(57) Abstract: The present invention discloses multi-stage bridge amplification methods which employs a double-stranded amplification product to initiate a subsequent stage of amplification. This double-stranded amplification product is formed during an amplification reaction and can be used to establish an iterative process. Using this double-stranded amplification product, the efficiency of bridge amplification increases significantly as a result of multiple stages of bridge amplification and permits, for example, the detection of a target molecule using a non-radioactive label (such as fluorescence).

METHODS FOR MULTI-STAGE SOLID PHASE AMPLIFICATION OF NUCLEIC ACIDS

RELATED APPLICATIONS

This application claims priority to United States Application Serial Number 09/513,300 filed February 25, 2000, the teachings of which are specifically incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

With an increasing appreciation for the molecular basis of numerous biological phenomenon through the advancement of molecular and recombinant technology, it is often highly desirable to examine many genetic elements within a single nucleic acid sample. Today it is possible to examine many genetic elements within a single nucleic acid sample using microarray technology. Currently, there exists technology in which probe arrays containing several hundred thousand oligonucleotides are present on a single glass chip (1 cm^2). (Wang, D.G., *et al.*, *Science*, 280:1077-1082 (1998)). Using microarray technology, the human genome can be explored and defined. For example, a microarray containing probes for 10^4 randomly distributed human single nucleotide polymorphism (SNPs) could be employed to generate a detailed genomic map of a single individual in a single hybridization experiment. This capacity, however, exceeds that achievable by polymerase chain reaction (PCR). Currently using PCR technology, it would be extremely difficult to amplify more than 100 independent loci in a single reaction. (Wang, D.G., *et al.*, *Science*, 280:1077-1082 (1998)).

Thus, there exists a need for novel methods that provide multiplex nucleic acid amplification. Preferably, these methods would make it possible to produce 10^3 to 10^4 unique products in a single reaction. This capacity would facilitate exploring entire genomes which in turn could be useful in the medical arts.

SUMMARY OF THE INVENTION

The present invention discloses methods for amplifying target nucleic acid molecules using a solid-phase amplification method. One such method is described in U.S. Patent No. 5,641,658, the teachings of which are incorporated by reference herein in its entirety. This single-stage solid-phase amplification method is referred to herein as "bridge amplification."

The present invention encompasses a multi-stage bridge amplification method which uses a recovered amplification product to initiate a second stage of bridge amplification. Subsequent stages of bridge amplification follow where each subsequent stage of bridge amplification is initiated with an amplification product produced in the previous stage of bridge amplification. This multi-stage method is recursive, and therefore provides for an iterative process whereby a single target molecule can be amplified over a hundred thousand-fold. This iterative process significantly increases the amplification power of bridge amplification.

More specifically, described herein is a solid-phase, multi-stage method for amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification. In the present method, double-stranded amplification products are produced during the first stage of bridge amplification which are subsequently used to initiate a second stage of bridge amplification, and double-stranded amplification products produced in the second stage of bridge amplification are used to initiate the third stage of bridge amplification, and so forth, through multi-stages of bridge amplification to produce amplified target molecules. Alternatively, single-stranded amplification products produced during the first stage of bridge amplification can be used to initiate second and subsequent stages.

The first stage of bridge amplification involves one, or more, target nucleic acid molecules mixed under conditions of hybridization with a solid support comprising immobilized oligonucleotide primers which are specific for the target molecules. For example, a sample (i.e., test sample) can contain a single type of target molecule and the solid support can comprise a pair of immobilized primers specific for that type of target molecule. Alternatively, the sample can contain multiple target molecules and the solid support will comprise multiple pairs of immobilized primers wherein each pair of primers are specific for one of the target molecules. The target molecules hybridize with their specific immobilized primers. The hybridization complexes that form are then subjected to amplification via thermocycle reactions, thus forming double-stranded amplification products. Amplification comprises approximately from about five to about fifty thermocycles, each thermocycle comprising denaturation, primer annealing and polymerization reactions (primer extension) carried out under conditions appropriate for each reaction. Typically, amplification comprises about thirty-five thermocycles.

The amplification products (e.g. double-stranded or single-stranded) are cleaved from the solid support. These newly released amplification products are then contacted with a fresh solid support comprising specific immobilized primers and initiate a second stage of bridge amplification. The stages of bridge amplification can be repeated until the desired amplification of the target molecule is achieved. The amplified target nucleic acid molecules can then be analyzed on the solid support, or they can be cleaved from the support for analysis by solution phase or solid phase methods.

The oligonucleotide primers of the present invention are immobilized to a solid support. These primers are specific for a given target nucleic acid molecule. Preferably, the primers are single-stranded DNA molecules. In one embodiment of the invention, a set of primers (e.g., a set of primers comprises a first and a second primer) specific for amplifying a target molecule is immobilized to a solid support. The first primer is complementary to a nucleotide sequence region contained within the target molecule, for example, the 3' terminal end. The second primer is complementary to the 3' terminal end of the complementary nucleic acid strand of

the target molecule. There are multiple sets of primers specific for various target molecules attached to the same solid support. Preferably, each primer contains at least one cleavable moiety. For example, the primers comprise a restriction site within their nucleotide sequence.

Preferably, the target molecule is a DNA molecule. Other nucleic acid molecules are within the scope of this invention, for example, RNA. The target nucleic acid molecule (or simply, target or target molecule) can originate from plant or animal tissue. Preferably, the target molecule contains one nucleotide sequence region that can hybridize to a first immobilized primer. The target molecule can be in a double-stranded or single-stranded form. If the presented target molecule is in a double-stranded form, then it is treated so as to render it into a single-stranded form.

The solid support can be beads, particles, sheets, dipsticks, rods, membranes, filters, fibers (e.g., optical and glass), and the like. Preferably, the solid support is a bead. The material composition of the solid support includes, but not limited to, plastic, nylon, glass, silica, metal, metal alloy, polyacrylamide, polyacrylate, crosslinked-dextran and combinations thereof. Preferably, the solid support is capable of being modified by the attachment of oligonucleotide primers.

Bridge amplification begins with a hybridization complex formed between a target molecule and a first oligonucleotide primer. (See FIG. 1). Preferably, the target molecule hybridizes to a first primer immobilized to the solid support, under conditions suitable for hybridization, thereby forming a hybridization complex. (See FIG. 1 a&b). The first primer is extended by the addition of deoxynucleotides under conditions suitable for polymerization. (See FIG. 1c). The newly formed duplex molecule comprising the target molecule hybridized to its complementary strand is subjected to denaturation, thereby releasing the target molecule from the duplex. The complementary strand remains attached to the solid support via the first primer. (See FIG. 1d).

The single-stranded complementary nucleic acid molecule forms a bridge hybridization complex by contacting a second primer which is immobilized to a solid support. (See FIG. 1e). Preferably, the second primer is immobilized to the same solid support as that to which the first primer is attached. The second primer is

extended by the addition of deoxynucleotides under conditions suitable for polymerization. (See FIG. 1f). This newly formed duplex molecule is subjected to denaturation yielding two single-stranded nucleic acid molecules attached to the solid support via their respective oligonucleotide primers. (See FIG. 1g). Each of the single-stranded nucleic acid molecules can form bridge hybridization complexes. (See FIG. 1h). Once a bridge hybridization complex is formed, nascent complementary strands are synthesized under conditions suitable for polymerization. (See FIG. 1i). This process (i.e., the themocycling process of steps "g" to "i" illustrated in FIG.1) is repeated from about five to about fifty times. Typically, amplification comprises about thirty-five thermocycles. Following this process, the duplex nucleic acid molecules are subjected to cleavage. (See FIG. 1j). In one embodiment, the first and second primers are cleaved using, for example, restriction endonucleases. This cleavage will sever the attachment of the duplex amplification product from its attachment to the solid support via the first and second primer. The released amplification product can be recovered and used to initiate a second round of bridge amplification. (See FIG. 2).

In one embodiment of the present invention, the detection of the presence or absence of one or more target molecules in a test sample using the multi-stage bridge amplification method is disclosed. In this embodiment, the amplification products can serve as signals, for example, to detect the presence or absence of a nucleic acid target in a biological sample, for example, microbial DNA. During the amplification process nascent amplification single-stranded nucleic acid molecules are formed by the incorporation of deoxynucleotides. One or more of these deoxynucleotides can be labeled prior to incorporation into the nascent amplification single-stranded nucleic acid molecules which then form the amplification products. The detection of one, or more, labeled nascent amplification products is indicative of the presence of at least one target molecule in a test sample. Labels other than radioactivity can be employed, such as chemiluminescence, luminescence and fluorescence.

In another embodiment of the present invention, a kit providing reagents for use in a solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification is disclosed.

In this kit the amplification products produced in the first stage of bridge amplification initiate a second stage of bridge amplification, and each subsequent stage of bridge amplification is initiated with an amplification product produced in the previous stage of bridge amplification, wherein one reagent comprises a solid phase support comprising a set of primers specific for one or more target nucleic acid molecules in quantity sufficient for at least two stages of bridge amplification.

Thus, the present invention provides methods for improved solid-phase amplification of target nucleic acid molecules. In particular, the present invention provides improved multiplex amplification methods for nucleic acid analysis.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1(a) - (j) is a schematic illustration of multi-stage bridge amplification.

FIG. 2 is a schematic illustration of progress from stage 1 of bridge amplification into stage 2 of bridge amplification.

FIG. 3(a) - (i) is a detailed schematic illustration of multi-stage bridge amplification.

FIG. 4 is the nucleotide sequence of the yeast LEU2 gene target, the PstI and XhoI restriction sites engineered into the 5'-terminal nucleotide sequences of the primers are not shown.

FIG. 5 is a photograph of a gel showing results obtained from performing a single-stage and a two-stage bridge amplification reaction.

DETAILED DESCRIPTION OF THE INVENTION

The present invention describes methods for amplifying target nucleic acid molecules using multiple stages of amplification employing a solid support. One such method that employs the use of a solid support is bridge amplification as described in U. S. Patent No. 5,641,658 to Adams and Kron, the teachings of which are incorporated herein by reference in its entirety. Essentially this methods consists of contacting a target molecule with a solid support to which are attached oligonucleotide primers specific for the target molecule. Hybridization occurs between the target molecule and immobilized oligonucleotide primer. In the

presence of appropriate amplification reagents, a complementary single-stranded nucleic acid molecule is synthesized using the target molecule as a template strand for polymerase extension of the immobilized primer to which the target molecule is hybridized forming a duplex amplification product. This duplex is denatured allowing for the target molecule to be released from the duplex. The complementary strand remains bound to the solid support via the immobilized oligonucleotide primer. This complementary strand forms a bridge-like structure by contacting another primer which is complementary to its 3'-terminal end. In the presence of appropriate reagents, a complementary strand is synthesized to the first complementary strand forming a duplex nucleic acid molecule. This duplex is denatured allowing for these complementary strands to contact and hybridize to fresh primers, thus facilitating new rounds of amplification.

Bridge amplification has a significant increase with respect to target capacity when compared to other amplification methods such as PCR. With the present invention, the amplification power using a multi-stage bridge amplification method approaches that of solution phase PCR.

Currently, large numbers of distinct target nucleic acid molecules contained within a single sample can be amplified using bridge amplification. (See U.S. Patent No. 5,641,658). Other multiplex methods are based upon solution phase PCR and, are limited to approximately 100 target nucleic acid molecules or less in a single reaction. This restriction presumably exists because the PCR primer sets are present at high concentrations and form unproductive "primer-dimer" products that are amplified more efficiently than the authentic amplification targets. (Chou *et al.*, *Nucleic Acid Res.*, 20:1717-1723 (1996), and Landegren, *Current Opinion in Biotech.*, 7:95-97 (1996)). The bridge amplification method obviates this restriction by using primer sets in which both primers for a particular target are immobilized to a common solid support. Unproductive primer-primer interactions are eliminated by primer immobilization. Moreover, amplification of each different target nucleic acid molecule occurs independently in a spatially delineated fashion. Spatial delineation occurs as a result of primer immobilization to any type of solid support. The primary function of immobilization is to eliminate unproductive interactions

between primers. Since immobilization to virtually any kind of surface (even beads) reduces the diffusion constant of primers significantly, it is not necessary to use ordered arrays to achieve the benefits of bridge amplification.

Despite the successes of bridge amplification, the method is not suitable for all applications requiring amplification technology. The success of PCR is due to the fact that it easily provides a million-fold amplification of a target nucleic acid molecule. This extent of amplification provides enough product for easy detection using inexpensive and safe fluorescence technologies. Single-stage bridge amplification as described by Adams and Kron (U.S. Patent No. 5,641,658), however, currently achieves approximately a several thousand-fold target amplification. Thus, single-copy human genes can only be detected in bridge amplification experiments if radioactivity is being employed as the detectable label.

Specifically encompassed in the present invention are methods for amplifying one, or more, target nucleic acid molecules using a multi-stage amplification strategy employing a solid support. The solid support comprises immobilized oligonucleotide primers. In each stage of bridge amplification, single target molecules are amplified several thousand-fold by generating double-stranded amplification nucleic acid molecules ("DSAM" also referred to herein as "double-stranded amplification products") which, following denaturation, hybridize with fresh immobilized primers (i.e., unused and unreactive primers) that are in the immediate vicinity of the original primer and are extended by a polymerase to generate new amplification products. One, or more, double-stranded amplification products are formed under suitable conditions by contacting (e.g., admixing) one, or more, target nucleic acid molecules with primers that are immobilized to a solid support and with amplification reagents (e.g., deoxynucleotides and DNA polymerase), such amplification reagents are well known to those of skill in the art. (Ausubel, F.M., *et al.,* (eds), Current Protocols in Molecular Biology, John Wiley & Sons (Pub.), vol. 2, ch. 15.4 (1991), the teachings of which are incorporated by reference herein in its entirety). (See FIG. 3).

A set of oligonucleotide primers (comprising a first and a second primer) is attached to a solid support. More than one set of primers designed for different

target nucleic acid molecules can be attached to the solid support, for simplicity only one set of primers specific for only one target molecule is described herein. Preferably, the attachment of the primers to the solid support is a covalent attachment. The oligonucleotide primers are preferably single-stranded DNA molecules. Preferably, a first primer is complementary to the single-strand of the target molecule used as a template for the amplification reaction forming the first double-stranded amplification product, whereas a second primer is complementary to the complementary strand of the target molecule. Both primers can have their 5'-terminal ends attached to the solid support, thus availing their 3'-terminal ends free to participate in the hybridization and primer extension reactions with the appropriate nucleic acid molecules. (U.S. Serial No. 08/812,105, and Rehman *et al.*, *Nucleic Acid Res.*, 27:649-655 (1999), the teachings of both of which are herein incorporated by reference in their entirety). The surface density of the primers is sufficiently high to allow the double-stranded amplification product of the reaction to span between the attached first and second primers in the form of a single or double-stranded nucleic acid bridge.

The oligonucleotide primers are attached to the solid support using covalent interactions. The oligonucleotide primers can have a range of from about 5 to about several hundred nucleotides (e.g., about 500 nucleotides) in length. Preferably, the primers can have a range of from about 5 to about 50 nucleotides in length. Most preferably, the primers can have a range from about 15 to about 30 nucleotides in length. The primers are designed based upon the target nucleic acid molecules desired to be amplified. The primer sets can be synthesized directly on the solid support, such as a bead support, using orthogonal protecting groups such as dimethyltrityl groups or levulinate (see Horn *et al.*, *Nucleic Acid Res.*,25:4835-4841 (1997); and Horn *et al.*, *Nucleic Acid Res.*,25:4842-4848 (1997), the teachings of which are incorporated herein by reference in their entirety), and phosphoramidite reagents and supports for performing 5' → 3' synthesis (see Glen Research catalog, 1998, Glen Research, Sterling, VA; and Coassin *et al.*, International Patent Application No. WO 94/24312, the teachings of which are herein incorporated by reference in their entirety). Alternatively, the primers can be synthesized using

standard methods and attached to the support postsynthetically. Methods for postsynthetic attachment of oligonucleotide primers are well known to those in the art. (See Rehman *et al., Nucleic Acid Res.*, 27:649-655 (1999), the teachings of which are herein incorporated by reference in its entirety).

Preferably, the oligonucleotide primers are synthesized such that a modified 5'-acrylamide moiety (Acrydite™ phosphoramidite, Mosaic Technologies, Boston, MA) is incorporated which will allow the primers to be immobilized within a solid support, for example, a solid support comprising acrylamide. Chemical or photochemical groups subject to cleavage are incorporated into the structure of the linker moieties on the support, or incorporated into one or both primers before immobilization. Additionally, it is possible to introduce cleavable groups during oligonucleotide synthesis in the form of modified phosphoramidites. (Olejnik *et al., Nucleic Acid Res.*, 26:3572-3576 (1998), the teachings of which are herein incorporated by reference in its entirety). The primers are preferably attached to the solid support using covalent interactions. (See Rehman *et al., Nucleic Acid Res.*, 27:649-655 (1999)). However, noncovalent attachment methods can also be practiced with this invention and are well known to those of ordinary skill in the art. (See Cass, T., and Ligler, F.S. (eds), "Immobilized Biomolecules in Analysis: A Practical Approach." 1998. Oxford University Press, Oxford, UK, the entire teachings of which are incorporated herein by reference).

The solid support can be beads, particles, sheets, dipsticks, membranes, filters, fibers (e.g., glass and optical), and the like. Preferably, the solid support is a bead. Suitable material compositions of the solid support includes, but not limited to, plastic, nylon, glass, silica, metal, metal alloy, polyacrylamide, polyacrylates, crosslinked-dextran and combinations thereof. Preferably, the solid support is capable of being modified by the attachment of oligonucleotide primers. The solid support can have any geometric shape. For example, the solid support can approximate a sphere (e.g., a bead). Alternatively, the solid support is planar as a sheet or membrane. The solid support can be magnetic. Preferably, the solid support is thermally stable (e.g., able to withstand temperatures of up to 100°C) to withstand thermocycling conditions typically used in PCR.

Typically, the target molecule is a DNA molecule. The target molecule can have a range of length of from about 30 to about 50,000 nucleotides in length. The target nucleic acid is either single or double-stranded. If the target molecule is in a double-stranded form, then it is subjected to denaturation resulting in two single-stranded nucleic acids. Both of these single-stranded nucleic acids individually can also be referred to as target molecules. For simplicity, only one strand will be discussed as a template strand that hybridizes to the first primer, thereby initiating the amplification reaction. However, it should be understood that the process is mirrored for the other target single-strand given an appropriate set of primers (i.e., same primers, but different order of interaction) immobilized to a solid support. Other nucleic acid molecules are also within the scope of this invention, for example, RNA. The target nucleic acid molecule (or simply, target or target molecule) can originate from plant or animal tissue, from a cell, tissue or organ culture system. Preferably, the target molecule has been purified prior to subjecting it to amplification. Methods of purifying nucleic acid are well known to those of ordinary skill in the art. (Ausubel, F.M., *et al.,* (eds), *Current Protocols in Molecular Biology*, John Wiley & Sons (Pub.), vol.1, ch. 2 through 4 (1991), the teachings of which are incorporated by reference herein in its entirety). Preferably, the target molecule, specifically the template strand, contains one nucleotide sequence region that can hybridize to a first immobilized primer.

A hybridization complex is formed by contacting (e.g., admixing) the target molecule with a first oligonucleotide primer, under conditions suitable for hybridization. (See U.S. Patent No. 5,641,658, and U.S. Serial No. 08/800,840, the teachings of which are herein incorporated by reference in their entirety). A single-stranded target molecule (i.e., the template strand) hybridizes to a first attached oligonucleotide primer. (See FIG. 3a). The first oligonucleotide primer is a primer that has a nucleotide sequence region that is complementary to a nucleotide sequence region contained within the template strand of the target molecule. The complementary region is from about 5 to about 50 nucleotides in length.

A first double-stranded amplification product (first "DSAM") is formed by contacting (e.g., admixing) the hybridization complex with amplification reagents

under conditions suitable for amplification. (See FIG. 3b). Under suitable amplification conditions, a nascent complementary strand is synthesized using the single-stranded target molecule as a template strand. A double-stranded amplification product ("DSAM") is formed following this amplification reaction and remains bound to the solid support. Conditions suitable for amplification comprise a thermally stable DNA Polymerase, deoxynucleotides, appropriate ionic strength and pH as well as other necessary reagents to facilitate a nucleic acid amplification reaction well known to those of ordinary skill in the art. (Ausubel, F.M., *et al.*, (eds), *Current Protocols in Molecular Biology*, John Wiley & Sons (Pub.), vol.2, ch. 15.4 (1991), the teachings of which are incorporated by reference herein in its entirety). The first primer is extended with deoxynucleotides forming a first single-stranded amplification nucleic acid molecule (first "SSAM" also referred to herein as "first single-stranded amplification molecule"). This first single-stranded amplification molecule is complementary to the target template and together they form the first double-stranded amplification product (first "DSAM").

The first double-stranded amplification product is a double-stranded nucleic acid comprising the target molecule hybridized to its complementary strand (i.e., the first single-stranded amplification molecule). Under denaturing conditions, the bound target nucleic acid molecule is separated from its bound complementary strand (i.e., the first single-stranded amplification molecule). (See FIG. 3c). The first single-stranded amplification molecule contacts the surface of the solid support and hybridizes to a complementary second oligonucleotide primer which contains a nucleotide sequence region complementary to the first single-stranded amplification molecule and is attached to the solid support, forming a first bridge hybridization complex (first "BHC"). (See FIG. 3d). The complementary region is from about 5 to about 50 nucleotides in length. Preferably, the solid support is the same support to which the first primer is attached.

A second double-stranded amplification product (second "DSAM") is formed under suitable amplification conditions where a nascent complementary strand is synthesized using the first single-stranded amplification molecule as the template strand. (See FIG. 3e). The second primer is extended with the addition of

deoxynucleotides such that a complementary strand to the first single-stranded amplification molecule is formed. This complementary strand is referred to as the second single-stranded amplification nucleic acid molecule (second "SSAM" also referred to herein as "second single-stranded amplification molecule"). The nucleotide sequence comprising this second single-stranded amplification molecule is a sequence that is identical to the original target molecule's nucleotide sequence. Hybridized together, the first and second single-stranded amplification molecules form the second double-stranded amplification product (second "DSAM").

The second double-stranded amplification product is subjected to denaturation. Denaturation is effectuated, for example, by placing the second double-stranded amplification product in an alkali environment (e.g., 15 mM NaOH). Alternatively, the double-stranded amplification product is subjected to melting temperatures which depend upon many factors such as the nucleotide base constituents. Suitable denaturing conditions are well known to those in the art. Following denaturation, the hydrogen bonds between the first and second single-stranded amplification molecules are broken resulting in first and second single-stranded amplification molecules. These single-stranded molecules still remain attached to the solid support via the oligonucleotide primers. The first single-stranded amplification molecule (first "SSAM") remains attached via the immobilized first primer; whereas, the second single-stranded amplification molecule (second "SSAM") remains attached via the immobilized second primer. (See FIG. 3f).

Under suitable denaturation/annealing conditions, multiple second bridge hybridization complexes (second "BHC") are formed. (See FIG 3g which illustrates, for simplicity, only two bridge hybridization complexes). For example, the attached first single-stranded amplification molecule contacts a fresh second oligonucleotide primer, which contains a complementary nucleotide sequence region to the 3'-end region of the first single-stranded amplification molecule, thus forming a second bridge hybridization complex. Similarly, the second single-stranded amplification molecule contacts a fresh first oligonucleotide primer which contains a complementary nucleotide sequence region to the 3'-end region of the second single-

stranded amplification molecule, thus forming another second bridge hybridization complex. Preferably, the oligonucleotide primers which are contacted by the first and second single-stranded amplification molecules are immobilized on the same solid support to which the single-stranded amplification molecules are attached.

Third and fourth double-stranded amplification products (third and fourth "DSAM") are formed by contacting the second bridge hybridization complexes with appropriate amplification reagents. (See FIG. 3h). The first and second immobilized primers are extended by the addition of deoxynucleotides. The extension of the second primer uses the first single-stranded amplification molecule as a template forming a third single-stranded nucleic acid amplification molecule (third "SSAM") also referred to herein as "third single-stranded amplification molecule"). The hybridized first and third single-stranded amplification molecules form a third double-stranded amplification product (third "DSAM"). In a similar manner, the extension of the first primer uses the second single-stranded amplification nucleic acid molecule as a template forming a fourth single-stranded amplification nucleic acid molecule (fourth "SSAM", also referred to herein as "fourth single-stranded amplification molecule"). The hybridized second and fourth single-stranded amplification molecules form a fourth double-stranded amplification product. This amplification thermocycle is typically repeated from about five to about fifty cycles generating multiple third and fourth double-stranded amplification products. More typically, amplification comprises about thirty-five thermocycles. Each cycle can consist of 95°, 60° and 72°C for about one minute duration for each temperature point. Such thermocycling conditions are well known to those skilled in the art. Additional rounds of thermocycling give rise to a multitude of additional amplification double-stranded products.

Amplification products ("AP") from the first stage of bridge amplification are released in a double-stranded form and are used to initiate the next stage of bridge amplification. (See FIG. 3i). The third and fourth double-stranded amplification products are completely cleaved from attachment to the solid support. (See FIG. 3i). Cleavage can be accomplished by either enzymatic or chemical means. Enzymatic cleavage is accomplished by incorporating a specific restriction

endonuclease site within the primers attached to the solid support. In one embodiment, the restriction site is the same in all of the attached primers. In another embodiment, the restriction site contained within the first primer is different from that restriction site contained within the second primer (e.g., the first primer contains a EcoRI site, while the second primer contains a HindIII site). Alternatively, a restriction site is incorporated in only one primer.

Methods for cleaving the double-stranded amplification molecule from the solid support other than restriction are also well known to those of skill in the art. For example, chemical cleavage is used if the two primers are attached to the solid support by a chemical linker that contains a chemically labile group. Dithiol linkages are one example of a linking chemistry that is heat stable but easily cleaved by chemical agents such as dithiothreitol (DTT), β-mercaptoethanol, Tris (2-carboxyethyl) phosphine HCl (TCEP) and other disulfide reducing agents. (Day *et al., Biochem. J.,* 278:735-740 (1991); Singh *et al., Methods in Enzymology,* 251:167-173 (1995), the teachings of which are herein incorporated by reference in its entirety). Alternatively, photochemical cleavage is employed if one or both of the two primers are attached to the solid support by a linkage moiety that is photochemically labile. Photochemical cleavable attachment chemistries for DNA oligonucleotides have been previously described. (Olejnik *et al., Nucleic Acid Res.,* 26:3572-3576 (1998) and U.S. Patent No. 5,679,773, the teachings of which are incorporated by reference herein in their entirety). For example, the photochemically cleavable linker can comprise a substituted nitrophenol group.

The released double-stranded products are recovered and applied to fresh amplification supports to initialize a second stage of bridge amplification. Subsequent stages of bridge amplification are initiated using double-stranded amplification products produced in the previous stage of bridge amplification.

Stages of bridge amplification can be repeated until a desired level of target molecule amplification is achieved. For example, two stages of bridge amplification can yield an amplification on the order of 10^6- fold (overall for two stages combined, 1000 x 1000; each stage producing about 1000-fold amplification). If a higher level

of amplification is desired, then more stages of bridge amplification can be performed.

Once the desired level of amplification has been achieved, the product formed on the solid support can be analyzed while still attached to the solid support. For example, if the solid support is a bead, the beads can be concentrated and analyzed for signal emission (such as fluorescence). Alternatively, the products can be cleaved from the support and analyzed by solution phase methods, for example, gel electrophoresis. (See the Exemplification).

Amplification products ("AP") can be detectably labeled during the polymerization reaction, for example, using labeled deoxynucleotides incorporated during the amplification process. The label can be radioactive, chemiluminescent, luminescent and fluorescent agents. Preferably, the label is a fluorescent agent. Direct labeling of the nucleic acid molecule of interest using modified nucleotides is accomplished by a number of enzymatic methods well known to those of ordinary skill in the art. (See Sambrook, *et al., Molecular Cloning: A Laboratory Manual,* 2nd edition, Cold Spring Harbor Press, Cold Spring Harbor, NY (1989), the teachings of which are incorporated by reference herein in its entirety). To detect an amplification product, an intercalating dye, such as ethidium bromide, can be used. Detection of a double-stranded amplification product can also be accomplished by employing a labeled nucleic acid comprising a nucleotide sequence region which is complementary to a nucleotide sequence region of the double-stranded amplification product which hybridizes to that molecule, for example, the third and/or fourth single-stranded amplification molecule.

In one embodiment of the present invention, a method for detecting the presence or absence of a target molecule in a test sample using the multi-stage bridge amplification method is described herein. Amplification products are formed during the amplification stages of the multi-staged bridge amplification method. These products can be labeled and detected as described in the preceding paragraph. (See above and Exemplification). Given the significant increase in amplification power, labels other than radioactivity can be used. Other labels which may be

employed in this detection scheme include chemiluminescence, luminescence and fluorescence. Preferably, the label is a fluorescent agent.

In another embodiment of the instant invention, a kit for use in a solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification is described. The amplification products produced in the first stage of bridge amplification initiate a second stage of bridge amplification, and each subsequent stage of bridge amplification is initiated with an amplification product produced in the previous stage of bridge amplification. One kit reagent comprises solid supports for performing at least two stages of bridge amplification. The solid supports comprise at least one primer set for amplifying one or more target molecules. For example, in one embodiment, the solid support reagent of the kit comprises beads wherein each bead comprises a set of primers specific for one or more target molecules.

The amplification power of solid-phase bridge amplification using this improved method of multi-stage bridge amplification described herein is significantly increased compared to single-stage bridge amplification. Each stage of amplification comprising from about 30 to about 40 amplification cycles can provide a several thousand-fold target amplification. The total extent of amplification over all of the stages is as high as the product of the individual amplification factors from each stage. For example, assuming a 1,000-fold amplification for a single stage of bridge amplification requiring thirty-five thermocycles, three consecutive stages of bridge amplification could yield an overall amplification as high as 10^9 (assuming perfect recovery and utilization of bridge amplification double-stranded molecules during each stage). Thus, this new method circumvents the problem of low amplification power of the original bridge method by the implementation of a multi-stage bridge amplification procedure which employs a double-stranded amplification product to initiate a second stage of bridge amplification.

The features and other details of the invention will now be more particularly described and pointed out in the exemplification. It will be understood that the particular embodiments of the invention are shown by way of illustration and not as

limitations of the invention. The principle features of this method are employed in various embodiments without departing from the scope of the invention.

EXEMPLIFICATION

Multistage Bridge Amplification

This example illustrates a two stage bridge amplification method using a yeast gene fragment (LEU2). The nucleotide sequence of yeast LEU2 gene, bases 7685 to 7943 (Genbank Accession No. AFO49063) is shown in Figure 4. The oligonucleotide primers were synthesized with a 5'-acrylamide modification (Acrydite™ phosphoramidite, Mosaic Technologies, Boston, MA) which allows the primers to be immobilized to a solid support, e.g. a polyacrylamide bead. Copolymerization of the modified primers with the acrylamide gel mix during bead fabrication produced a solid support with primers immobilized on and within the bead. The primers remained stably attached via the 5'-acrylamide groups during thermocycling. (Rehman *et al., Nucleic Acid Res.*, 27:649-655 (1999)).

Acrylamide beads with immobilized primers were prepared by pipetting 1 μL drops of a solution containing 10% polyacrylamide (acrylamide/bis, 29:1), 10 mM sodium borate buffer (pH 8.0), 100 μM of each 5'-acrylamide primer (Leu2F2.Pst: 5'-QTT TTT TTT T*CT GCA G*AA CCG TGG CAT GGT TC-3' [SEQ ID No. 1], and Leu2R3.Xho: 5'QTT TTT TTT T*CT CGA G*CT GTG GAG GAA ACC ATC AAG-3' [SEQ ID No. 2] (restriction sites are italicized, "Q" represents a 5'-acrylamide group) and 0.2% ammonium persulfate (wt/vol) into degassed mineral oil containing 0.4% N,N,N',N'-tetramethylethylenediamine (TEMED). Polymerization was allowed to proceed for thirty minutes at room temperature. Excess mineral oil was decanted and the beads were transferred to a 50 mL disposable tube containing 30 mL of TE buffer (10 mM Tris-HCl (pH 8.0) and 1 mM EDTA). The remaining mineral oil was extracted 2 to 3 times using chloroform (15 mL per extraction). The beads were then washed with several rounds of TE buffer (15 mL per round). To remove non-immobilized primers from the beads, the preparation was equilibrated with 0.5 x TBE (89 mM Tris-borate (pH 8.3) and 2 mM

EDTA) and placed into the wells of a vertical polyacrylamide gel, subsequently the preparation was subjected to electrophoresis for sixty minutes at 20 V/cm.

Prior to hybridization the beads were subjected to 15 thermocycles in the absence of DNA Polymerase to remove primers that were not thermally stable. The beads were then equilibrated for 1 to 2 hours with 1 x thermopol buffer (10 mM KCl, 20 mM Tris-HCl (pH 8.8), 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, New England Biolabs, Beverly, MA), plus 50 μg/mL bovine serum albumin (BSA) and 120 ng/mL denatured *E. coli* genomic DNA. Thermocycling was performed using a profile of thirty seconds each at 94°C, 60°C and 72°C.

Bridge Amplification on Bead Supports

Hybridization and amplification were performed in separate steps. Each amplification reaction utilized a single 1 μL bead support. The yeast (*Saccharomyces cerevisiae*) target DNA was restricted using two restriction endonucleases that do not cut within the desired amplification target nucleic acid sequence (Sau96 and HincII, New England Biolabs, Beverly, MA). Hybridization reactions contained 1 primer-modified bead, 1 x thermopol buffer, 50 μg/mL BSA and 50 μg/mL restricted yeast DNA in a total reaction volume of 100 μL. Reactions were initiated by a two minute denaturation at 94°C, and hybridization was carried out for thirty minutes at 60°C in a shaking microplate incubator (Taitec Microincubator M-36, Taitec Instruments, San Jose, CA). After hybridization, the beads were washed once in 100 μL of 1 x thermopol buffer with 50 μg/mL BSA at 60°C with shaking for 10 minutes.

First Stage Bridge Amplification

Following hybridization, the beads were transferred to a 30 μL amplification reaction mixture containing 1 x thermopol buffer, 50 μg/mL BSA, 200 μM each dATP, dCTP, dGTP and dTTP, and 0.01 U/μL Vent DNA Polymerase (New England Biolabs, Beverly, MA). The reactions were incubated at 72°C for five minutes to extend hybridized target molecules. After the initial extension, target molecules bound to the beads were amplified through 35 thermocycles consisting of thirty seconds each at 94°C, 60°C and 72°C.

Restriction Cleavage of Amplified Products

Following amplification, the beads were rinsed once using 1 x NEB buffer 3 (50 mM Tris-HCl (pH 7.9 at 25°C), 10 mM $MgCl_2$, 1 mM DTT) containing 0.1 mg/mL BSA. Products were then restricted from the beads using XhoI and PstI in combination. Alternatively, first-stage products were restricted from the beads using PstI alone. Restriction reactions were performed in a 30 μL volume containing 1 x NEB buffer 3, 0.1 mg/mL BSA, and 30 units of each restriction endonuclease (New England Biolabs, Beverly, MA). The restriction endonucleases XhoI and PstI cut within the 5'-terminal nucleotide sequences of the oligonucleotide primers. Restriction was carried out for three hours at 37°C.

Second Stage Bridge Amplification and Analysis

Following restriction digestion of the first stage, 10 μL of the doubly-restricted product were used as the input target nucleic acid molecule for the next stage of bridge amplification. Singly-restricted product was eluted from the PstI treated beads by heating at 94°C for two-minutes. Ten μL of the eluted product was used as the input target nucleic acid molecule for the next stage of bridge amplifications. The next stage (in this case, the second stage) of hybridization and amplification was performed as that previously described above for the first stage reaction. Products were restricted from the second stage solid supports with RsaI and EcoRI using the same buffer and method described for cleavage of the first stage products. RsaI and EcoRI cleaved within the double-stranded amplification product inside the position of the primers. Ten μL aliquots of each reaction were subjected to electrophoresis in a non-denaturing 1 x TBE, 10% polyacrylamide gel (Novex, San Diego, CA). The gel was stained with SYBR green I (Molecular Probes, Eugene, OR) and imaged using a Molecular Dynamics Fluorimager 595 (Molecular Dynamics, Sunnyvale, CA). (See FIG.5).

Figure 5 illustrates the gel that was obtained from performing the experiment. Each of the lanes 3 through 6 were loaded with the double-stranded amplification products obtained from a single independent bridge amplification reaction. In each case, 10 μL, or 33% of a total 30 μL restriction reaction were loaded. The products shown in lanes 3 and 4 were from PstI-XhoI double digestion

of a first stage amplification reaction. Lanes 5 and 6 show products from second stage reactions in which single-stranded first stage amplification products were used as the input target nucleic acid molecules for the second stage. Lanes 7 and 8 show products from second stage amplification reactions in which double-stranded first stage amplification products were used as input targets (45-fold increase over the level of first-stage amplification). Lane "m" contains DNA size markers: 0.05 μg of an MspI digest of pBR322 (New England Biolabs, Beverly, MA).

Fluorimetric analysis of the products shown in lanes 7 and 8 show a 15-fold increase over the level of the first stage bridge amplification products (lanes 3 and 4). Lanes 5 and 6 show products from second-stage amplification reactions where single-stranded first stage amplification products were used as input targets (45-fold increase over the level of first-stage amplification). Thus, the use of a multi-stage bridge amplification improves the overall extent of amplification.

While this invention has been particularly shown and described with references to preferred embodiments thereof, it will be understood by those skilled in the art that various changes in form and details may be made therein without departing from the spirit and scope of the invention as defined by the appended claims.

CLAIMS

What is claimed is:

1. A solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification, wherein amplification products produced in the first stage of bridge amplification initiate second and subsequent stages of bridge amplification.

2. The method of Claim 1, wherein each stage of bridge amplification comprises the steps of:

   (a) hybridizing target nucleic acid molecules to a set of oligonucleotide primers immobilized on a solid support, and

   (b) thermocycling to amplify the target nucleic acid molecules hybridized to the oligonucleotide primers forming an amplification product.

3. The method of Claim 2, wherein the thermocycling step (b) comprises about 5 to about 50 thermocycles.

4. A solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising tow or more stages of bridge amplification, wherein double-stranded amplification products produced in the first stage of bridge amplification initiate second and subsequent stages of bridge amplification.

5. The method of Claim 4, wherein each stage of bridge amplification comprises the steps of:

   (a) hybridizing target nucleic acid molecules to a set of oligonucleotide primers immobilized on a solid support, and

(b) thermocycling to amplify the target nucleic acid molecules hybridized to the oligonucleotide primers forming a double-stranded amplification product.

6. The method of Claim 5, wherein the thermocycling step (b) comprises about 5 to about 50 thermocycles.

7. The method of Claim 5 further comprising the steps of:

(c) cleaving the double-stranded amplification product;

(d) recovering the double-stranded amplification product of step (c);

(e) contacting the double-stranded amplification product with a fresh solid support, and

(f) repeating steps (a) through (e) to amplify the target nucleic acid molecules.

8. The method of Claim 7 further comprising step (g) wherein the amplified target nucleic acid molecules are analyzed on the solid support.

9. A method of amplifying one, or more, target nucleic acid molecules using a solid support comprising two or more immobilized oligonucleotide primers which specifically hybridize to the target nucleic acid molecule, comprising the steps of:

(a) hybridizing target nucleic acid molecules to a set of oligonucleotide primers immobilized on a solid support;

(b) thermocycling to amplify the target nucleic acid molecules hybridized to the oligonucleotide primers forming a double-stranded amplification product;

(c) cleaving the double-stranded amplification product;

(d) recovering the double-stranded amplification product of step (c);

(e) contacting the double-stranded amplification product with a fresh solid support, and

(f) repeating steps (a) through (e) to amplify the target nucleic acid molecules.

10. The method of Claim 9, wherein the thermocycling step (b) comprises about 5 to about 50 thermocycles.

11. The method of Claim 10, wherein each thermocycle comprises about one minute each at 95°C, 60°C and 72°C.

12. A method of amplifying one, or more, target molecules using a solid support comprising two or more immobilized primers which specifically hybridize to the target molecule, comprising the steps of:

(a) forming a hybridization complex comprising a single-stranded target nucleic acid molecule hybridized to a first oligonucleotide primer immobilized to the solid support, comprising contacting the target nucleic acid molecule with the solid, under conditions suitable for hybridization;

(b) forming a first amplification product comprising the target nucleic acid molecule hybridized to a first amplification molecule, by contacting the hybridization complex of step (a) with amplification reagents, under conditions suitable for a primer-mediated amplification reaction, wherein a first amplification molecule is formed by extending a first primer with deoxynucleotides to form a complementary strand to the target nucleic acid molecule, thereby forming an amplification product;

(c) denaturing the amplification product of (b), thereby releasing the single-stranded target nucleic acid molecule from the first amplification product;

(d) forming a first bridge hybridization complex comprising the first amplification molecule of step (b) and a second oligonucleotide

primer immobilized to the solid support, under conditions suitable for hybridization, thereby forming the first bridge hybridization complex;

(e) forming a second amplification product by contacting the hybridization complex of step (d) with amplification reagents, under conditions suitable for a primer-mediated amplification reaction, wherein a single-stranded second amplification molecule is formed that is complementary to and hybridizes with the first amplification molecule of step (d), thereby forming a second amplification product;

(f) denaturing the second amplification product of (e), wherein the hybridization between the first and second amplification molecules is disrupted, thereby releasing a single-stranded first and second amplification molecule;

(g) forming second bridge hybridization complexes comprising
(i) the first amplification molecule of step (f) and a second oligonucleotide primer immobilized to the solid support, under conditions suitable for hybridization, and
(ii) the second amplification molecule of step (f) and a first oligonucleotide primer immobilized to the solid support, under conditions suitable for hybridization, thereby forming the set of second bridge hybridization complexes;

(h) forming a third and fourth amplification product by contacting the hybridization complexes of step (g) with amplification reagents, under conditions suitable for a primer-mediated amplification reaction, wherein
(i) the third amplification product is formed comprising a nascent single-stranded third amplification molecule that is complementary to and hybridizes with the first amplification molecule, and
(ii) the fourth amplification product is formed comprising a nascent single-stranded fourth amplification molecule that is complementary to and hybridizes with the second amplification molecule, thereby forming the third and fourth amplification products;

(i) cleaving one, or more, first and second oligonucleotide primers, thereby cleaving the third and fourth amplification products completely from attachment to the solid support;

(j) applying released amplification products from step (i) to fresh solid supports comprising unused immobilized oligonucleotide primers, and

(k) repeating steps (a) through (j) one, or more, additional stages, wherein the released third and fourth amplification product from step (i) is used as the target nucleic acid molecule in step (a).

13. The method of Claim 12, wherein the first and second primers are immobilized to the solid support through one, or more, covalent interactions.

14. The method of Claim 12, wherein steps (f), (g) and (h) are repeated from about 5 to about 50 times.

15. The method of Claim 12, wherein the oligonucleotide primers are immobilized via co-polymerization with a polymeric surface layer on the solid support.

16. The method of Claim 12 wherein in step (i), the third and fourth amplification products are cleaved from the solid support using chemical means.

17. The method of Claim 16, wherein the chemical means is a reducing agent.

18 The method of Claim 17, wherein the reducing agent is selected from the group consisting of: dithiothreitol, β-mercaptoethanol and TCEP.

19. The method of Claim 12 wherein in step (i), the oligonucleotide primers are cleaved from the solid support using photochemical means.

20. The method of Claim 19, wherein the oligonucleotide primers comprise a nitrophenol group.

21. The method of Claim 12 wherein in step (i), the third and fourth amplification products are cleaved from solid support using enzymatic means.

22. The method of Claim 21, wherein the enzymatic means is accomplished using one, or more, restriction endonucleases.

23. The method of Claim 12, wherein the denaturant for the amplification products is selected from the group consisting of: high temperature, high pH, organic solvent, chaotropic agents and combinations thereof.

24. The method of Claim 12, wherein the material composition of the solid support is selected from the group consisting of: plastic, glass, silica, nylon, metal, metal alloy, polyacrylamide, polyacrylate, crosslinked-dextran and combinations thereof.

25. The method of Claim 24, wherein the solid support is a bead.

26. The method of Claim 12 wherein the bead comprises one, or more, oligonucleotide primer sets specific for a particular target molecule.

27. The method of Claim 12, wherein the first and second oligonucleotide primers are from about 5 to about 500 nucleotides in length.

28. The method of Claim 12, wherein one or more amplification products are labeled.

29. The method of Claim 28, wherein the label is selected from the group consisting of: radioactivity, chemiluminescence, luminescence and fluorescence.

30. A solid-phase, multi-stage method of detecting the presence or absence of one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification, wherein amplification products produced in the first stage of bridge amplification initiate second and subsequent stages of bridge amplification, and each subsequent stage of bridge amplification is initiated with amplification products produced in the previous stage of bridge amplification.

31. The method of Claim 30, wherein one or more amplification products are labeled.

32. The method of Claim 31, wherein the label is selected from the group consisting of: radioactivity, chemiluminescence, luminescence and fluorescence.

33. A solid-phase, multi-stage method of detecting the presence or absence of one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification, wherein double-stranded amplification products produced in ;the first stage of bridge amplification initiate second and subsequent stages of bridge amplification, and each subsequent stage of bridge amplification is initiated with double-strand amplification products produced in the previous stage of bridge amplification.

34. The method of Claim 33, wherein one or more amplification products are labeled.

35. The method of Claim 34, wherein the label is selected from the group consisting of: radioactivity, chemiluminescence, luminescence and fluorescence.

36. The method of Claim 33, wherein each stage of bridge amplification comprises the steps of:

(a) hybridizing target nucleic acid molecules to a set of oligonucleotide primers immobilized on a solid support, and

(b) thermocycling to amplify the target nucleic acid molecules hybridized to the oligonucleotide primers by the formation of bridge amplification double-stranded molecules.

37. The method of Claim 33, wherein the thermocycling step (b) comprises about 5 to about 50 thermocycles.

38. The method of Claim 33 further comprising the steps of:

(c) cleaving the double-stranded amplification product;

(d) recovering the double-stranded amplification product

(e) contacting the double-stranded amplification product with a fresh solid support, and

(f) repeating steps (a) through (e) to amplify the target nucleic acid molecules.

(g) detecting the presence of the target molecules, wherein the detection of the single-stranded or double-stranded amplification molecules is indicative of the target molecules in a test sample.

39. A method of detecting the presence or absence of one, or more, target nucleic acid molecules using a solid support comprising two or more immobilized oligonucleotide primers, of which at least one primer specifically hybridizes to the target nucleic acid molecules, comprising the steps of:

(a) hybridizing target nucleic acid molecules to a set of oligonucleotide primers immobilized on a solid support;

(b) thermocycling to amplify the target nucleic acid molecules hybridized to the oligonucleotide primers forming double-stranded amplification products;

(c) cleaving the double-stranded amplification products;

(d) recovering the double-stranded amplification products of step (c),

(e) contacting the double-stranded amplification products with a fresh solid support, and

(f) repeating steps (a) through (e) to amplify the target nucleic acid molecules, and

(g) detecting the presence of the target molecules, wherein the detection of one or more double-stranded amplification products is indicative of the target molecules in a test sample.

40. The method of Claim 39, wherein the thermocycling step (b) comprises about 5 to about 50 thermocycles.

41. The method of Claim 40, wherein each thermocycle comprises about one minute each at 95°C, 60°C and 72°C.

42. The method of Claim 39, wherein one or more double-stranded amplification products are labeled.

43. The method of Claim 42, wherein the label is selected from the group consisting of: radioactivity, chemiluminescence, luminescence and fluorescence.

44. A kit for use in a solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification, wherein amplification products produced in the first stage of

bridge amplification initiates a second stage of bridge amplification, and each subsequent stage of bridge amplification is initiated with an amplification product produced in the previous stage of bridge amplification, wherein one reagent comprises solid phase supports comprising at least one set of primers for amplifying at least one target nucleic acid molecule, the supports provided in a quantity sufficient for at least two stages of bridge amplification.

45. The kit of Claim 44, wherein the solid support reagent comprises beads wherein each bead comprises a set of primers specific for one or more target molecules.

46. A kit for use in a solid-phase, multi-stage method of amplifying one, or more, target nucleic acid molecules comprising two or more stages of bridge amplification, wherein double-stranded amplification products produced in the first stage of bridge amplification initiates a second stage of bridge amplification, and each subsequent stage of bridge amplification is initiated with a double-stranded amplification product produced in the previous stage of bridge amplification, wherein one reagent comprises solid phase supports comprising at least one set of primers for amplifying at least one target nucleic acid molecule, the supports provided in a quantity sufficient for at least two stages of bridge amplification.

47. The kit of Claim 46, wherein the solid support reagent comprises beads wherein each bead comprises a set of primers specific for one or more target molecules.

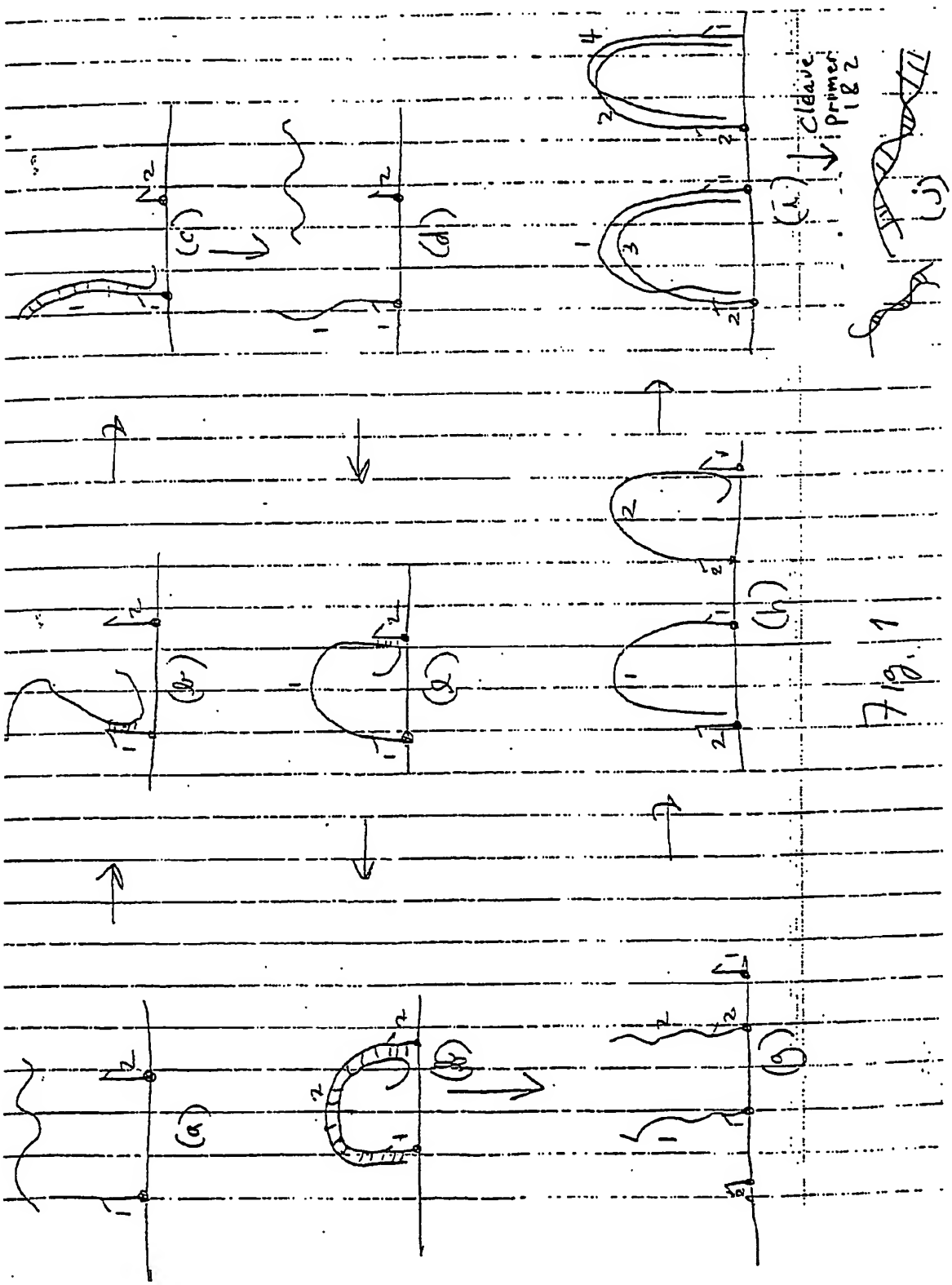

Fig. 1

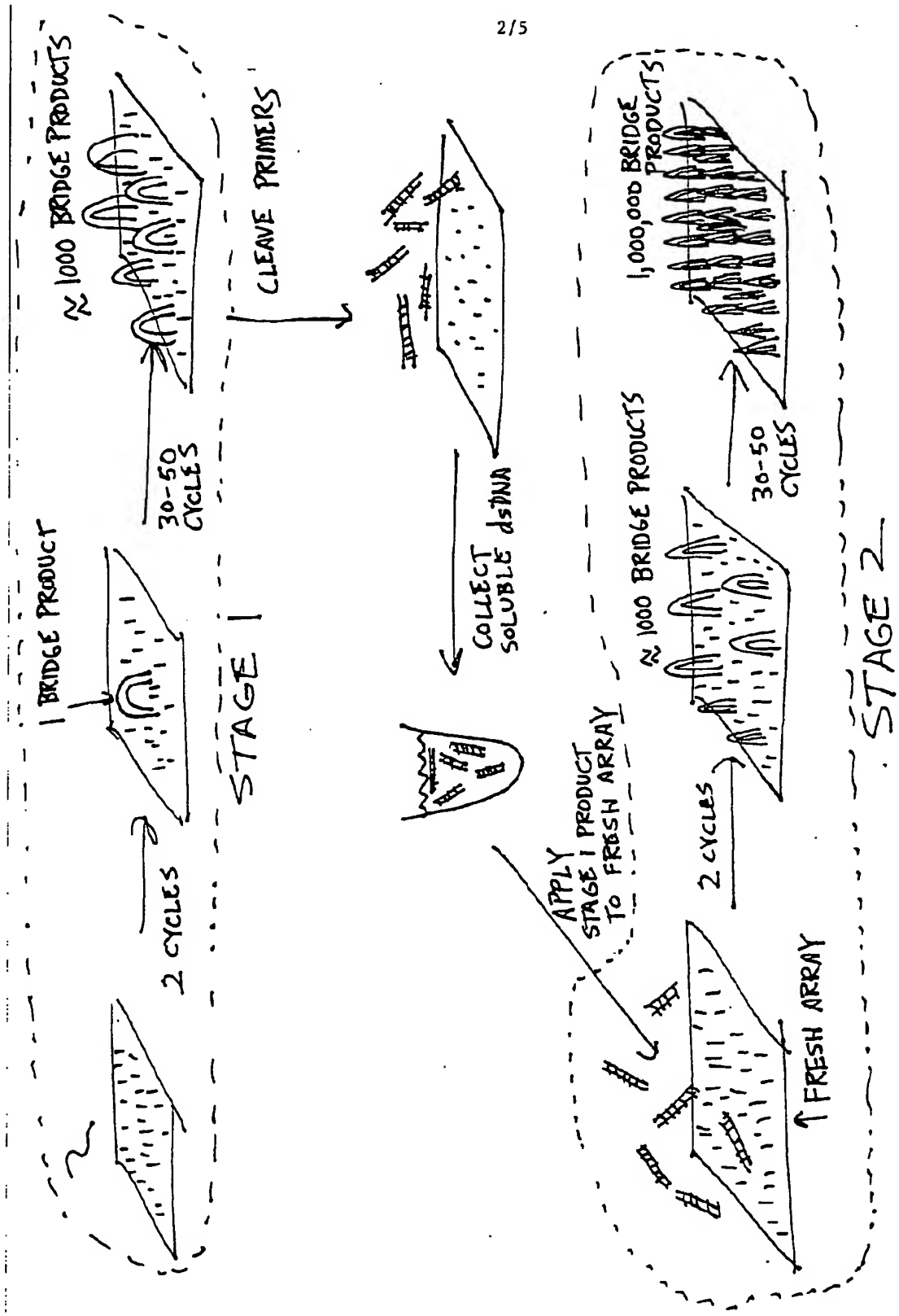
1 BRIDGE PRODUCT
2 CYCLES
STAGE 1
≈ 1000 BRIDGE PRODUCTS
30-50 CYCLES
CLEAVE PRIMERS
COLLECT SOLUBLE dsDNA
APPLY STAGE 1 PRODUCT TO FRESH ARRAY
FRESH ARRAY
2 CYCLES
≈ 1000 BRIDGE PRODUCTS
30-50 CYCLES
1,000,000 BRIDGE PRODUCTS
STAGE 2

Fig. 2

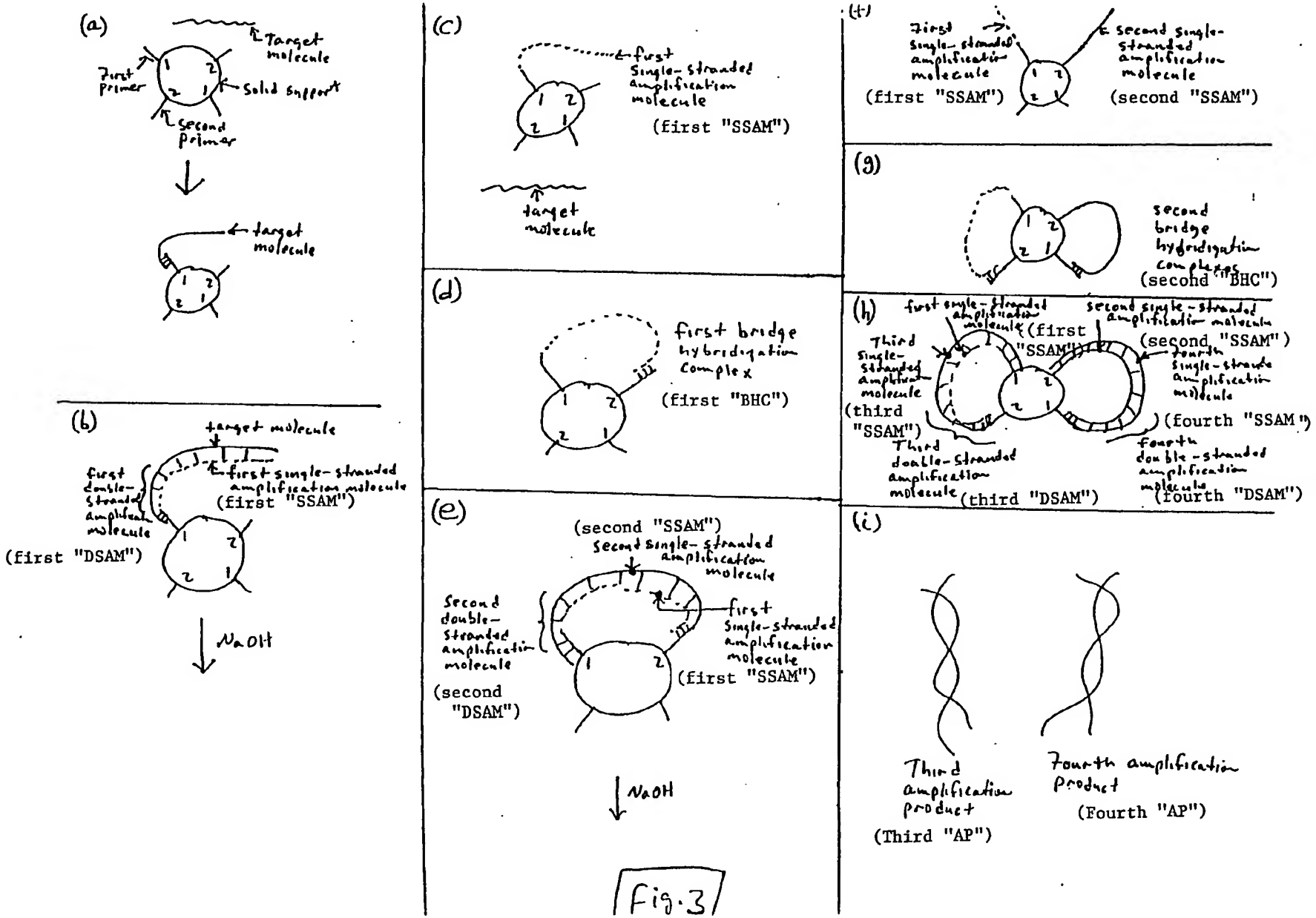
(a)
Target molecule
First primer
Solid support
Second primer
target molecule
(b)
target molecule
first single-stranded amplification molecule
(first "SSAM")
first double-stranded amplification molecule
(first "DSAM")
NaOH
(c)
first single-stranded amplification molecule
(first "SSAM")
target molecule
(d)
first bridge hybridization complex
(first "BHC")
(e)
(second "SSAM")
second single-stranded amplification molecule
second double-stranded amplification molecule
(second "DSAM")
first single-stranded amplification molecule
(first "SSAM")
NaOH
(f)
first single-stranded amplification molecule
(first "SSAM")
second single-stranded amplification molecule
(second "SSAM")
(g)
second bridge hybridization complexes
(second "BHC")
(h)
first single-stranded amplification molecule
(first "SSAM")
second single-stranded amplification molecule
(second "SSAM")
Third single-stranded amplification molecule
(third "SSAM")
fourth single-stranded amplification molecule
(fourth "SSAM")
Third double-stranded amplification molecule
(third "DSAM")
fourth double-stranded amplification molecule
(fourth "DSAM")
(i)
Third amplification product
(Third "AP")
Fourth amplification product
(Fourth "AP")

Fig. 3

```
gcagaaccgt ggcatggttc gtacaaacca aatgcggtgt tcttgtctgg
---------------------> RsaI
Leu2f2 primer

caaagaggcc aaggacgcag atggcaacaa acccaaggaa cctgggataa

cggaggcttc atcggagatg atatcaccaa acatgttgct ggtgattata

ataccattta ggtgggttgg gttcttaact aggatcatgg cggcagaatc

aatcaattga tgttgaacct tcaatgtagg gaattcgttc ttgatggttt
cctccacag                          EcoRI    <-----------
---------
Leu2R3 primer
```

Fig. 4

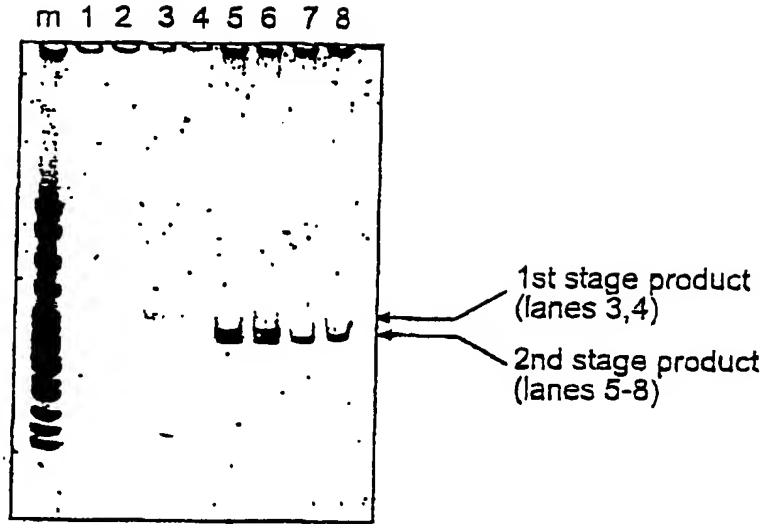
m 1 2 3 4 5 6 7 8
1st stage product
(lanes 3,4)
2nd stage product
(lanes 5-8)

Figure 5